

Development of Two PCR-Based Techniques for Detecting Helical and Coccoid Forms of *Helicobacter pylori*

M. Shahamat,* M. Alavi, J. E. M. Watts, J. M. Gonzalez,† K. R. Sowers, D. W. Maeder, and F. T. Robb

Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, Maryland 21202

Received 24 December 2003/Returned for modification 12 February 2004/Accepted 11 April 2004

The primary mode of transmission of *Helicobacter pylori*, a human pathogen carried by more than half the population worldwide, is still unresolved. Some epidemiological data suggest water as a possible transmission route. *H. pylori* in the environment transforms into a nonculturable, coccoid form, which frequently results in the failure to detect this bacterium in environmental samples by conventional culture techniques. To overcome limitations associated with culturing, molecular approaches based on DNA amplification by PCR have been developed and used for the detection of *H. pylori* in clinical and environmental samples. Our results showed the *glmM* gene as the most promising target for detection of *H. pylori* by PCR amplification. Under optimal amplification conditions, *glmM*-specific primers generated PCR-amplified products that were specific for *H. pylori* and some other *Helicobacter* species. Genome sequence analysis revealed the existence of a conserved region linked to a hypervariable region upstream of the 16S rRNA gene of *H. pylori*. Selective PCR primer sets targeting this sequence were evaluated for the specific detection of *H. pylori*. One primer set, Cluster2 and B1J99, were shown to be highly specific for *H. pylori* strains and did not produce any PCR products when other *Helicobacter* species and other bacterial species were analyzed. In tests with 32 strains of *H. pylori*, 6 strains of other *Helicobacter* species, 8 strains of *Campylobacter jejuni*, and 21 strains belonging to different genera, the primers for *glmM* were selective for the *Helicobacter* genus and the primers containing the region flanking the 16S rRNA gene were selective for *H. pylori* species only. The combination of two sensitive PCR-based methods, one targeting the *glmM* gene and the other targeting a hypervariable flanking region upstream of the 16S rRNA gene, are complementary to each other. Whereas the *glmM*-specific primers provide a rapid, sensitive presumptive assay for the presence of *H. pylori* and closely related *Helicobacter* spp., the primers for sequences flanking the 16S rRNA gene can confirm the presence of *H. pylori* and locate the potential source of this bacterium.

Helicobacter pylori is a gram-negative microaerophilic bacterium that infects human gastric epithelial cell surfaces and the overlying gastric mucin, which is a highly specialized niche. This bacterium is carried by more than half of the human population worldwide and is an important pathogen. It is the major cause of peptic ulcers and a contributor to other illnesses, ranging from childhood malnutrition to gastric cancer, and is known to be an agent causing susceptibility to other food- and waterborne pathogens (7, 10, 11, 39, 46).

Infection with *H. pylori* occurs worldwide, but the geographical prevalence varies greatly (41). Higher infection rates occur in developing countries, where 80% of middle-aged adults are infected compared with infection rates of only 20 to 50% in developed countries (16, 22, 42). Worldwide, the occurrence of *H. pylori* infection increases with age, and specific ethnic groups are also at greater risk (12, 19, 27, 31).

There is evidence that countries with poor sanitation have greater infection rates, which suggests a common water source as the reservoir rather than person-to-person transmission (1). There is a strong correlation between the consumption of

municipal water and *H. pylori* infection in some countries (42). Recent data suggest that the bacterium is present in river water and drinking water (23, 24). However, the exact mode of *H. pylori* transmission remains largely unknown, as proof of a waterborne or food-borne route of transmission for *H. pylori* requires its detection from environmental reservoirs, foodstuffs, and water contaminated with human fecal material.

It has been observed that *H. pylori* cells can transform in vitro from a culturable spiral-shaped form to an as-yet nonculturable coccoid form. This change in morphology is accompanied by physiological changes that make it very difficult to recover the bacterium by routine culturing methods. These changes have been monitored in water microcosms in our laboratory (30, 38) and by other researchers (20, 45).

To overcome this problem, a variety of culture-independent methods have been tested for the detection of *H. pylori* in clinical samples (7, 9, 25, 36). PCR-based methods have also been used to detect *H. pylori* from environmental samples (1, 5, 15, 37). Most of these PCR methods target the 16S rRNA gene, random chromosome sequences, the 26-kDa species-specific antigen gene, the urease A (*ureA*) gene, and the urease C (*ureC*) gene (5, 28). Among these PCR methods, PCR-based detection of the *ureC* gene appears to be the most promising for detection of *H. pylori* (28). The *ureC* gene, renamed *glmM* by De Reuse et al. in 1997 (13), encodes phosphoglucosamine mutase, an enzyme catalyzing the interconversion of glu-

* Corresponding author. Mailing address: Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, MD 21202. Phone: (410) 234-8881. Fax: (410) 234-8896. E-mail: shahamat@umbi.umd.edu.

† Present address: Instituto de Recursos Naturales y Agrobiología, Consejo Superior de Investigaciones Científicas, 41080 Seville, Spain.

cosamine-6-phosphate into glucosamine-1-phosphate, which is subsequently transformed into *N*-acetylglucosamine (13). Although PCR amplification of *glmM* can detect *H. pylori*, we determined that it also detected a number of other *Helicobacter* species. Therefore, a complementary technique was needed to discriminate *H. pylori* specifically from other *Helicobacter* species.

Analysis of the complete genomic sequence from two strains of *H. pylori*, strains J99 and 26695 (2, 44) (GenBank accession numbers AE001439 and AE000511, respectively), has revealed that the 16S rRNA and 23S rRNA genes are not contiguous in the *H. pylori* chromosome. In all other species analyzed, the 16S and 23S rRNA genes are concatenated in their genome sequence. The regions flanking the 16S and 23S rRNA genes of *H. pylori* were examined with the aim of identifying specific sequences that could be used as molecular markers to identify *H. pylori* in environmental samples.

The goal of the research described herein was to design and validate an accurate and sensitive molecular method for rapid detection of both the helical and coccoid forms of *H. pylori* from culture, clinical, and environmental samples.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and sample preparation. The *H. pylori* strains and other bacterial strains from diverse genera used to establish assay specificity are listed in Table 1. Thirty-two strains of *H. pylori* were obtained from the National Collection of Type Cultures (Public Health Laboratory, London, United Kingdom), American Type Culture Collection, and our own collection, which included isolates from patients in local hospitals. Six strains of other species of *Helicobacter*, 8 strains of *Campylobacter jejuni*, and 21 strains belonging to different bacterial genera were included in this study (Table 1).

Growth conditions and media for all strains used in this study are shown in Table 2. The cultures were sequentially transferred twice prior to testing. To induce the coccoid form of *H. pylori*, a freshly prepared suspension of *H. pylori* cells was inoculated into a series of prewashed 2-liter flasks, with each flask containing 1 liter of sterile drinking water, well water, and river water. The initial concentration of the cells was 10^7 to 10^8 cells/ml. Flasks were incubated at 15 and 4°C. At established intervals, samples were removed aseptically from the microcosms, cultured on blood agar plates for viable count, and simultaneously counted using the acridine orange direct count method of Hobbie et al. (21) with some modifications. Morphological and viability changes in the cells were monitored. After 20 to 30 days, 99% of the cells in the water microcosms converted into the coccoid form (30, 38).

DNA extraction. DNA was extracted from 1-ml aliquots of sample (from culture or water microcosms) with the Fast DNA SPIN kit (Bio 101, Vista, Calif.) per the manufacturer's instructions. Briefly, the samples were added to lysing reagents in a matrix tube that contained silica and ceramic beads of various sizes. The cells were lysed in a Bio 101 Savant FastPrep bead beater (Savant, Farmingdale, N.Y.) for 30 s at 5.5 m s^{-1} and then centrifuged for 30 min at $10,000 \times g$. The supernatant was transferred to a tube containing a protein-precipitating solution and centrifuged for 10 min at $10,000 \times g$. The supernatant was removed, combined with the DNA-binding matrix, and centrifuged through a spin filter. The filter was then washed with a salt-ethanol solution and centrifuged. After the filter was dry, the DNA on the spin filters was eluted in 100 μl of sterile water (Sigma) and then purified by electrophoresis on a 1.3% (wt/vol) low-melting-point agarose gel. Chromosomal DNA was excised from the gel and recovered with a Promega Wizard PCR prep kit (Promega, Madison, Wis.), according to the manufacturer's instructions.

PCR primers and conditions. (i) **PCR primers for *glmM* amplification.** Oligonucleotides that specifically hybridize *H. pylori* genes encoding phosphoglucosamine mutase (*glmM*) and targeting conserved consensus sequences from clinical strains (28) were used in PCR amplification reactions. The primers used in this study are presented in Table 3. All PCR amplifications were performed using the GeneAmp PCR kit with *Taq* DNA polymerase (Perkin-Elmer, Inc.) in a PTC200 thermal cycler (MJ Research, Watertown, Mass.). The PCR conditions described by Lu et al. in 1999 (28) were further optimized as follows: an initial denaturation step of 2 min at 95°C, followed by 30 cycles, with 1 cycle

TABLE 1. Bacterial species used in this study

Species	Strain	Source ^a
<i>H. pylori</i>	11637	NCTC
<i>H. pylori</i>	33098	PHLS
<i>H. pylori</i>	62815	PHLS
<i>H. pylori</i>	12954	PHLS
<i>H. pylori</i>	20200	PHLS
<i>H. pylori</i>	60190	PHLS
<i>H. pylori</i>	12648	PHLS
<i>H. pylori</i>	12654	PHLS
<i>H. pylori</i>	33097	PHLS
<i>H. pylori</i>	11639	NCTC
<i>H. pylori</i>	26694	PHLS
<i>H. pylori</i>	95e	PHLS
<i>H. pylori</i>	7546	PHLS
<i>H. pylori</i>	TX30a	PHLS
<i>H. pylori</i>	43526	ATCC
<i>H. pylori</i>	43504	ATCC
<i>H. pylori</i>	11219	PHLS
<i>H. pylori</i>	RSB6	PHLS
<i>H. pylori</i>	A314	PHLS
<i>H. pylori</i>	26695	PHLS
<i>H. pylori</i>	52816	PHLS
<i>H. pylori</i>	MS-AN19	LH
<i>H. pylori</i>	MS61	LH
<i>H. pylori</i>	MS-PA18	LH
<i>H. pylori</i>	MS-RAY	LH
<i>H. pylori</i>	MS-HO25	LH
<i>H. pylori</i>	MS-HO26	LH
<i>H. pylori</i>	MS168	LH
<i>H. pylori</i>	MS52	LH
<i>H. pylori</i>	MS-CO49	LH
<i>H. pylori</i>	MSa54	LH
<i>H. pylori</i>	MS-UM2	LH
<i>P. putida</i>	17428	ATCC
<i>M. jannaschii</i>	JAL-1	OCM
<i>H. mustelae</i>	43772	ATCC
<i>H. felis</i>	49179	ATCC
<i>H. pametensis</i>	51478	ATCC
<i>H. nemestrinae</i>	49396	ATCC
<i>H. muridarum</i>	49282	ATCC
<i>H. canis</i>	51401	ATCC
<i>C. jejuni</i>	33560	ATCC
<i>C. jejuni</i>	MS4	LF
<i>C. jejuni</i>	MS3	LF
<i>C. jejuni</i>	MS2	LF
<i>C. jejuni</i>	MS1	LF
<i>C. jejuni</i>	194	LH
<i>C. jejuni</i>	89	LH
<i>C. jejuni</i>	81	LH
<i>E. coli</i>	K-12	LS
<i>E. coli</i>	HU735	PHLS
<i>E. coli</i>	O157-H7	USDA
<i>V. cholerae</i> O1	14035	ATCC
<i>V. vulnificus</i>	27562	ATCC
<i>Salmonella</i> serotype Typhimurium	221	USDA
<i>Shigella dysenteriae</i> type 1	14731	CIB
<i>B. subtilis</i>	168	USDA
<i>Bacillus stearothermophilus</i>	7953	ATCC
<i>P. aeruginosa</i>	43495	ATCC
<i>A. hydrophila</i>	1028	WR
<i>A. hydrophila</i>	1728	WR
<i>A. caviae</i>	681	WR
<i>A. jandaei</i>	396	WR
<i>R. pickettii</i>	27512	ATCC
<i>R. pickettii</i>	27511	ATCC
<i>R. pickettii</i>	49129	ATCC
<i>B. diminuta</i>	19146	ATCC
<i>Acinetobacter</i> spp.	MS	RW

^a Abbreviations: NCTC, National Collection of Type Culture, London, United Kingdom; PHLS, Public Health Laboratory Service, London, United Kingdom; ATCC, American Type Culture Collection; LH, isolated from biopsy or stool samples from patients in local hospitals in Maryland; LF, isolated from chicken swabs from a local chicken farm in Maryland; LS, laboratory strain; CIB, clinical isolate from the International Center for Diarrhoeal Disease Research in Bangladesh; WR, isolate from a water reservoir, School of Public Health, University of Sao Paulo, Sao Paulo, Brazil; RW, isolate from river water in our laboratory; OCM, *Methanocaldococcus jannaschii* JAL-1 was obtained from the Oregon Collection of Methanogens (OCM 168 = DSM 2661).

TABLE 2. Scheme for growth of bacterial species used in this study

Species	Media used	Growth at the following temp. (°C):	Conditions	Incubation time (days)
<i>H. pylori</i>	Columbia agar + B ^a	37	Microaerophilic ^b	2–3
<i>Helicobacter</i> spp. ^c	Columbia agar + B	37	Microaerophilic	3–4
<i>C. jejuni</i>	Columbia agar + B	41	Microaerophilic	2
<i>Aeromonas</i> spp.	LB agar (Miller) ^d	30	Aerobic	2
<i>M. jannaschii</i>	Specific medium ^e	83	Anaerobic	2
<i>Acinetobacter</i> sp.	TSA ^f	30	Aerobic	2
<i>B. stearothersophilus</i>	TSA	55	Aerobic	2
Other spp. ^g	TSA	37	Aerobic	2

^a Columbia agar (Oxoid) with 7% defibrinated horse blood.
^b Under microaerophilic conditions using Oxoid 3.5-liter jars with BBL Campy Pak Plus (Becton Dickinson).
^c Six other species of *Helicobacter* were grown under the above conditions (strains given in Table 1).
^d Luria-Bertani agar (Miller) contains 10 g of sodium chloride/liter.
^e Cultivation procedure was performed by the method of Mukhopadhyay et al. (35).
^f TSA, trypticase soy agar (Difco).
^g *Escherichia coli*, *Vibrio cholerae* O1, *Vibrio vulnificus*, *Shigella dysenteriae*, *Salmonella* serotype Typhimurium, *Ralstonia pickettii*, *Brevundimonas diminuta*, and *Bacillus subtilis*.

consisting of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. A final extension step of 5 min at 72°C concluded the reaction. For controls, the 16S rRNA gene sequence was amplified using universal primers.

(ii) **PCR primers for the 16S rRNA hypervariable flanking region.** The PCR primers Cluster2 and B1J99 (Table 3) target sequences that flank a hypervariable region upstream of a 16S rRNA gene in *H. pylori*. These primers were designed on the basis of information from the complete genome sequences of two *H. pylori* strains J99 and 26995 (GenBank accession numbers AE001439 and AE000511, respectively). A 16S rRNA gene sequence from *H. pylori* strain 26695 was used as a query using the BLAST algorithm (3) against *Helicobacter* genomic sequences to find the number and location of the 16S rRNA gene sequences in the two published *H. pylori* genomes. The graphical interfaces GeneMate and Cross (<http://comb5-156.umbi.umd.edu/genemate/getseq.html>) were used to visualize BLAST results. PCR was performed by a hot start *Taq* polymerase (Qiagen, Valencia, Calif.) in a final reaction volume of 50 µl with 200 µM deoxynucleoside triphosphate and 0.4 µM concentration of each primer using genomic DNA as the template. Amplification conditions used to amplify the hypervariable region flanking the 16S rRNA gene were as described above except that an annealing temperature of 60°C and longer extension steps (1 min at 72°C) were used.

To determine the sensitivities of intergenic spacer region (ISR) primers, DNA extracted from *H. pylori* reference strain 11637 was serially diluted, and 1 µl of each dilution was used in the PCR. The starting concentration of DNA as determined by absorbance at 260 nm was 157 ng/µl. Serial dilutions were made by mixing equal volumes of the DNA and ultrapure water in consecutive tubes, with the final dilution having an estimated concentration of 0.5 pg/µl.

Cloning and sequencing of the PCR products. The PCR amplicons from the region flanking the 16S rRNA gene were cloned into *Escherichia coli* by using a TA-PCR cloning kit (Invitrogen) according to the manufacturer's instructions. The recombinant plasmids were purified using a miniprep plasmid purification kit (Qiagen) and sequenced using an ABI 377 DNA sequencer (Applied Biosystems, Foster City, Calif.). The DNA fragment between the primers Cluster2 and B1J99 was sequenced using M13 reverse and T7 promoter sequencing primers and the additional primers Hp2R and Hp1F. The primers Hp2R and Hp1F were based on the sequences downstream of the Cluster2 and B1J99

sequences, respectively. The sequences were then aligned using the computer multiple-sequence-aligning program ClustalW (43).

RESULTS

Two sets of primers were used for PCR amplification to identify *H. pylori* in cultures and water microcosms.

PCR primers for the *glmM* gene. A total of 26 strains of *H. pylori* were tested with the *glmM* primers, and PCR results were positive for all strains tested. DNA was extracted from helical and coccoid forms of strains 52185, 61, 33097, and 11637 in spiked water. Other *Helicobacter* spp., including *H. mustelae*, *H. felis*, *H. pametensis*, and *H. nemestrinae*, were also tested. Two species (*H. mustelae* and *H. nemestrinae*) were positive, and the other two were negative with the *glmM* primers. Four strains of *C. jejuni* and 18 strains of other microbial species, including three strains of *Ralstonia pickettii* (27512, 27511, and 49129), three strains of *E. coli* (K-12, HU735 isolated from human blood, and O157-H7), two vibrio strains (*Vibrio cholerae* O1 and *Vibrio vulnificus*), two *Pseudomonas* species (*Pseudomonas aeruginosa* and *Pseudomonas putida*), two strains of *Aeromonas hydrophila* (1028 and 1728), two other species of *Aeromonas* (*Aeromonas caviae* and *Aeromonas jandaei*), one strain of *Bacillus subtilis*, one strain of *Shigella* (*Shigella dysenteriae*), one strain of *Salmonella* (*Salmonella enterica* serotype Typhimurium), one strain of *Methanocaldococcus* (*M. jannaschii*), and one strain of *Acinetobacter* tested negative with the *glmM* primers (Fig. 1). *H. pylori* also tested

TABLE 3. Primer sequences used in this study

Primer	Target site	Sequence (5'→3')	Reference
<i>glmM</i> -Forward	Phosphoglucosamine mutase gene	AGG CTT TTA GGG GTG TTA GGG GTT T	28
<i>glmM</i> -Reverse		AAG CTT ACT TTC TAA CAC TAA CGC	28
519F	16S rRNA gene	CAG CMG CCG CGG TAA TWC	26
1406R		ACG GGC GGT GTG TRC	26
Cluster2	16S rRNA hypervariable flanking region of <i>H. pylori</i>	GGC GTT ATC AAC AGA ATG GC	This study
B1J99		CTC AGT TCG GAT TGT AGG CTG C	This study
Hp2R		CTC CTT TCT AGA GAA AAG CTT	This study
Hp1F		TGT TTG GTG GGC TAT GGT T	This study

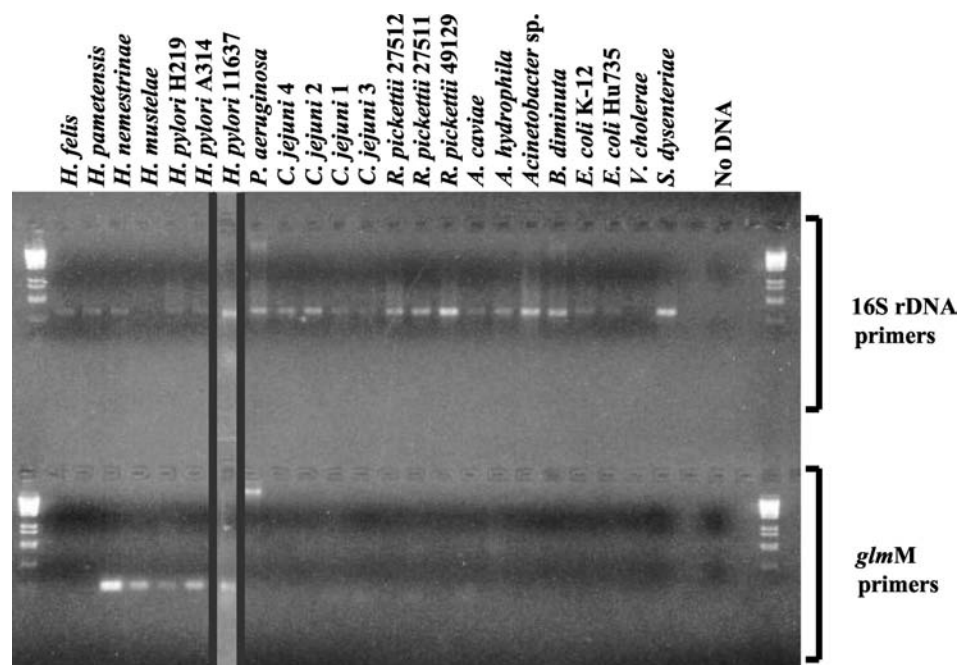


FIG. 1. Representative results for PCR products generated from a broad spectrum of bacteria, closely related strains of *Campylobacter*, and selected species of *Helicobacter*, using primers for the *H. pylori glmM* gene. PCR conditions are described in the text. All samples were amplified with universal primers 519F and 1406R as positive controls.

positive in a laboratory-prepared cocktail that included two strains of *E. coli*, *Vibrio*, *Bacillus*, *Salmonella*, and *Shigella* spp. suspended in filtered river water and incubated at 15°C. The PCR had a sensitivity of 0.1 pg of *H. pylori* DNA as previously reported (28).

PCR of *H. pylori* unique 16S rRNA-flanking region. Two primers were designed to amplify a unique hypervariable region upstream the 16S rRNA gene of *H. pylori*. These primers were tested with DNA extracted from *H. pylori*, other *Helicobacter* species, and a number of other species of bacteria (Table 1). A positive PCR signal was detected for all 32 strains of *H. pylori* tested. In contrast, *H. mustelae*, *H. felis*, *H. pametensis*, *H. muridarum*, *H. canis*, and *H. nemeistrinae* tested negative with these PCR primers. The PCR primers also did not hybridize with DNA from 28 other strains including eight strains of *C. jejuni* (from American Type Culture Collection, local hospital and local farms) three strains of *Ralstonia pickettii* (27512, 27511, and 49129), three strains of *E. coli* (K-12, HU735 isolated from human blood, and O157-H7), two strains of vibrios (*V. cholerae* O1 and *V. vulnificus*), two species of *Pseudomonas* (*P. aeruginosa* and *P. putida*), two strains of *A. hydrophila* (1028 and 1728), two other species of *Aeromonas* (*A. caviae* and *A. jandaei*), one strain of *B. subtilis*, one strain of *B. stearothermophilus*, one strain of *Salmonella*, one strain of *Shigella*, and one strain of *Acinetobacter* (Fig. 2). The selectivity of the assay was further confirmed by detection of *H. pylori* in a laboratory-prepared cocktail spiked with a mixture of *H. pylori*, *Escherichia coli* strains, *Vibrio* spp., *Bacillus* spp., *Salmonella* spp., and *Shigella* spp.

The sensitivities of primers specific for the hypervariable region of *H. pylori* DNA flanking the 16S rRNA gene were tested with serial dilutions of genomic DNA extracted from *H.*

pylori type strain 11637. The lower limit of detection was 2 pg of *H. pylori* DNA as found by the method used in this experiment (Fig. 3).

DISCUSSION

At this time, there are more than 23 *Helicobacter* species (6, 14), and more species are being identified. Some of these non-*H. pylori* species are found in clinical samples, and others colonize animals (18). This diversity of *Helicobacter* species creates a challenge for the detection of *H. pylori* from environmental waters. The aim of this study was to develop a specific method for rapid detection of *H. pylori* and to be able to selectively identify *H. pylori* in mixed microbial communities.

The detection of *H. pylori* is critical due to the high prevalence of this species, its worldwide distribution, and the large number of individuals carrying this species (7, 10, 11, 16, 38, 41, 46). In addition, *H. pylori* cells have the ability to form resistant, coccoid forms. *H. pylori* cells in the coccoid stage cannot be cultured and can be detected only by culture-independent strategies (5, 38).

H. pylori-specific probes that target the 16S rRNA, urease (*ureA*), and phosphoglucosamine mutase (*glmM*) (4, 28) genes have been proposed. The 16S ribosomal DNA and *ureA* primers did not detect all of the *Helicobacter* spp. under the reaction conditions described (data not shown). Results with a wide spectrum of bacteria tested in this study showed that the primers for *glmM* were selective for *Helicobacter* spp. and did not amplify products from non-*Helicobacter* spp.

In order to determine whether the assay was selective for *Helicobacter* spp., DNA from other species of *Helicobacter* and the closely related genus *Campylobacter* was tested under the

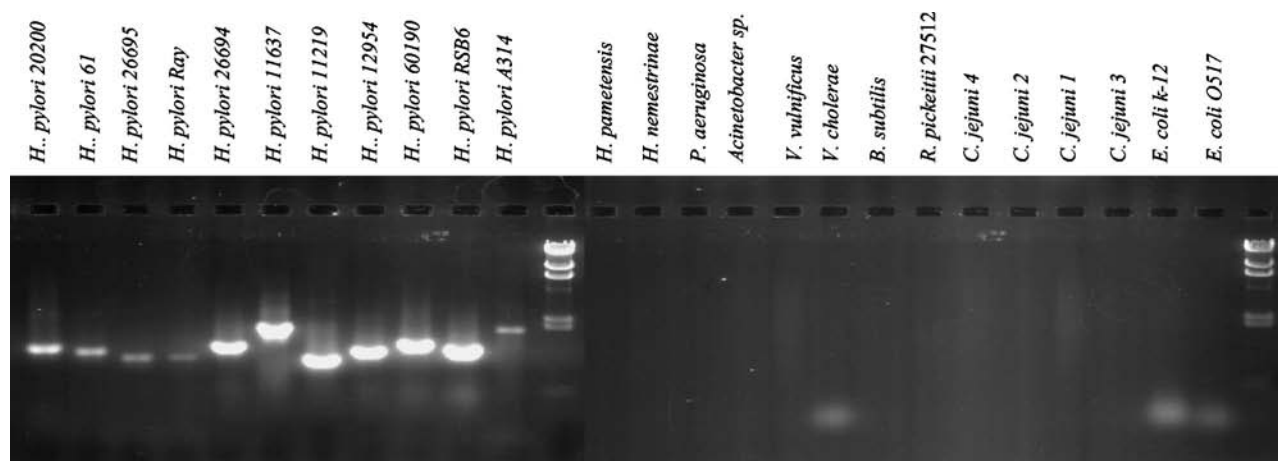


FIG. 2. Representative results for PCR using the unique hypervariable region of *H. pylori* flanking the 16S rRNA gene. PCR using primer pair (Cluster2 and B199) resulted in a high specificity for detecting *H. pylori* strains. This figure shows 11 positive lanes for *H. pylori*, two negative lanes for two other species of *Helicobacter*, four negative lanes for *C. jejuni*, and eight negative lanes for other bacterial species tested.

same conditions. Initial results showed that although the assay detected all *Helicobacter* species, *Campylobacter* species were also detected (data not shown). Reaction conditions were modified to develop a more stringent assay specific for *Helicobacter* spp. as described in Materials and Methods. After this optimization, results showed that only *Helicobacter* spp. were detected, and several environmental strains of the closely related genus *Campylobacter* no longer yielded amplification products (Fig. 1). The PCR-based method targeting *glmM* was previously proposed by Lu et al. in 1999 (28), but they did not report whether the PCR method was tested on any other *Helicobacter* species. Bickley et al. (4) found that this PCR test was specific to *H. pylori* and did not yield any products when tested on five other species of *Helicobacter*. We found that this PCR assay using the same primers yielded positive results for *H. pylori* and some other *Helicobacter* spp. tested.

To overcome this limitation, another set of PCR-based strategies was evaluated to identify *H. pylori* from cultures and

water microcosms. The target for this PCR was a unique sequence flanking a 16S rRNA gene sequence in *H. pylori*.

The ISR between the 16S and the 23S rRNA genes has been the target for designing species- and even strain-specific molecular probes. This has been possible due to the high inter- and intraspecific variability (8, 17, 33, 34, 40). The advantage of this region is that the flanking 16S and 23S rRNA genes at the 5' and 3' ends of the ISR contain highly conserved sequences (i.e., the 16S and 23S rRNA genes). Thus, conserved primers can be used for the selective amplification of ISR from bacterial species. However, in *H. pylori*, a ribosomal 16S rRNA gene is not followed in the genome sequence by the 23S rRNA gene. The rRNA operon structure of *H. pylori* has an atypical bacterial rRNA operon structure, as 16S rRNA sequences are physically removed from the 23S and 5S rRNA sequences and positioned on different DNA strands. There are two sets of rRNA genes in the genomes of *H. pylori* strains. The 16S rRNA genes for strain J99 are located on the plus strand at positions

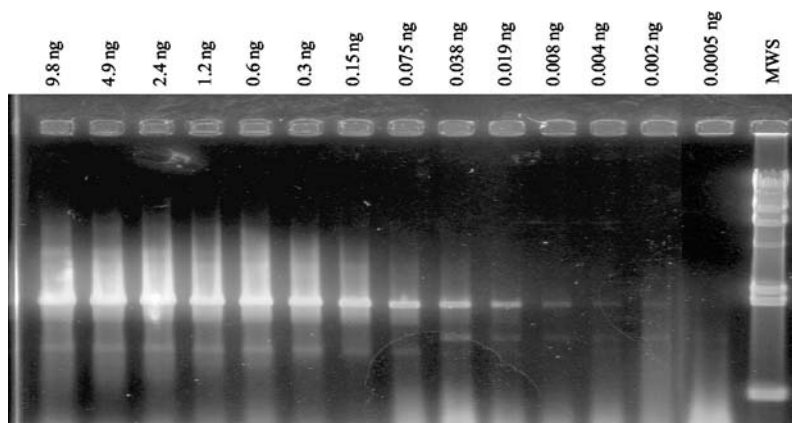


FIG. 3. The sensitivity of primers specific for the hypervariable region flanking the 16S rRNA gene for the detection of *H. pylori* DNA was tested with serial dilutions of genomic DNA extracted from *H. pylori* type strain 11637. The starting concentration of DNA as determined spectrophotometrically was 157 ng/ μ l. Serial dilutions were made by mixing equal volumes of the DNA and ultrapure water in consecutive tubes, with the final dilution having an estimated concentration of 0.5 pg/ μ l.

1188029 to 1189529 and 1463047 to 1464547, and the 16S rRNA genes for strain 26995 are located on the plus strand at positions 1207583 to 1209081 and 1511137 to 1512634. The associated primary transcript is synonymous with the minus strand. These regions lack the context of the typical hypervariable ISR of the rRNA operon found in other bacteria. The intervening region is poorly conserved. This pairing is specific for one of the copies of the 16S rRNA gene, and no suitably conserved region was identified for the other copy of the gene.

A nonstandard ISR is present in *H. pylori*. Nevertheless, a highly variable region, likely a remnant of its primitive ISR, is followed by a short conserved sequence which could be used for the design of a diagnostic probe for PCR amplification.

We designed a primer pair specific for *H. pylori* (Cluster2 and B199 primers) and evaluated its specificity in discriminating non-*H. pylori* species while uniquely identifying *H. pylori*. The first primer (B1J99) overlaps the 16S rRNA sequence and is synonymous with the primary transcript to avoid hybridization with 16S rRNA. The second (Cluster2) represents a sequence conserved between *H. pylori* J99 and *H. pylori* 26695, about 1,000 bases upstream on the opposite strand. We have sequenced and compared this conserved region from a number of *H. pylori* strains. The length of this region ranges from 992 to 1,548 bp, depending on the *H. pylori* strain.

Experimental testing of the PCR primer pair resulted in a high specificity for detecting *H. pylori* strains. PCR amplification targeting this hypervariable region showed positive results for every *H. pylori* strain tested and negative results for every *Helicobacter* species (non-*H. pylori* species) and other bacterial species tested. These results suggest that the proposed primer set resulted in a specific detection strategy for *H. pylori* from cultures, clinical, and environmental samples. In addition, the proposed PCR detection strategy for *H. pylori* using the 16S rRNA hypervariable region complements the results obtained by PCR amplification with *glmM*-specific primers. While the use of *glmM*-specific primers allows the detection of *Helicobacter* species (both *H. pylori* and other species of the genus), PCR amplifications based on the proposed primers specific for the region flanking the 16S rRNA gene of *H. pylori* discriminate the non-*H. pylori* species of this genus. Furthermore, sequence comparison using the proposed hypervariable region upstream of the 16S rRNA gene of *H. pylori* can be an easy way to distinguish *H. pylori* strains, since these sequences are highly specific for each *H. pylori* strain. For this purpose, these sequences have been deposited in the GenBank DNA database (accession no. AY505018 to AY505034, AY505037 to AY505043, and AY505045) and they are available for homology searches using BLAST (3) in future applications and rapid identification methods.

In addition, PCR amplification using the proposed primers allows detection not only of *H. pylori* but also of *H. pylori* strains, as the amplified product can be used to differentiate strains of this species. Review of the literature has not revealed other published studies that used PCR amplification targeting the proposed hypervariable region upstream of the 16S rRNA gene in *H. pylori*. The specificity and sensitivity of these primers, particularly the primers containing the region flanking the 16S rRNA gene, will significantly help to accurately and rapidly detect *H. pylori* in environmental waters, including drinking water, well water, and river water. This is the first report

proposing the use of this DNA sequence for the rapid detection and identification of *H. pylori* in both helical and coccoid forms. Recent published data indicate that *H. pylori* can be detected in different water samples (5, 15, 20, 29, 32). Culturing techniques are not an option for most of these samples due to conversion of the bacterium to the nonculturable coccoid form. Although the PCR detection system does not distinguish viable from nonviable bacteria in any given sample, our combination PCR-based approach provides the effective presumptive assay for determining the presence of *H. pylori* in cultures and in environmental water samples on the same day. Since the coccoid form of *H. pylori* represents a persistent form which can exist in water for long periods of time, positive results obtained by using these probes could indicate the presence of such cells.

ACKNOWLEDGMENTS

This research was funded in part by EPA's Science to Achieve Results (STAR) program grant R82803701. J.M.G. is supported by a contract from the Spanish Ministry of Science and Technology (Ramon y Cajal program).

REFERENCES

1. Akcan, Y., E. S. Alper, M. Z. Bicik, and N. Aytug. 2000. The transmission of *Helicobacter pylori* via exposure to common sources outweighs the person-to-person contact among spouses in developing countries. *Am. J. Gastroenterol.* **95**:317–319.
2. Alm, R. A., L. S. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust. 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature* **397**:176–180.
3. Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
4. Bickley, J., R. J. Owen, A. G. Fraser, and R. E. Pounder. 1993. Evaluation of the polymerase chain reaction for detecting the urease C gene of *Helicobacter pylori* in gastric biopsy samples and dental plaque. *J. Med. Microbiol.* **39**:338–344.
5. Bunn, J. E., W. G. MacKay, J. E. Thomas, D. C. Reid, and L. T. Weaver. 2002. Detection of *Helicobacter pylori* DNA in drinking water biofilms: implications for transmission in early life. *Lett. Appl. Microbiol.* **34**:450–454.
6. Cantet, F., C. Magras, A. Marais, M. Federighi, and F. Megraud. 1999. *Helicobacter* species colonizing pig stomach: molecular characterization and determination of prevalence. *Appl. Environ. Microbiol.* **55**:4672–4676.
7. Chanto Grettel, A. O., N. Gras, R. A. Alm, F. Megraud, and A. Maralis. 2002. Identification of strain-specific genes located outside the plasticity zone in nine clinical isolates of *Helicobacter pylori*. *Microbiology* **148**:3671–3680.
8. Christensen, H. J. K., and J. E. Olsen. 1999. Differentiation of *Campylobacter coli* and *C. jejuni* by length and DNA sequence of the 16S-23S rRNA internal spacer region. *Microbiology* **145**:99–105.
9. Chuanfu, L. L., H. A. Thanzhu, D. S. Chi, D. A. Ferguson, C. Jiang, J. J. Laffan, and E. Thomas. 1997. Differentiation of *Helicobacter pylori* strains directly from gastric biopsy specimens by PCR-based restriction fragment length polymorphism analysis without culture. *J. Clin. Microbiol.* **35**:3021–3025.
10. Clemens, J., A. M. Rao, M. Qadri, F. Huda, S. B. Kay, F. P. Van Loon, D. Sack, B. A. Pradhan, and R. B. Sack. 1995. Impact of infection by *Helicobacter pylori* on the risk and severity of endemic cholera. *J. Infect. Dis.* **171**:1653–1656.
11. Dale, A., T. J. Darboe, M. K. Coward, W. A. Harding, and L. T. Weaver. 1998. *Helicobacter pylori* infection, gastric acid secretion, and infant growth. *J. Pediatr. Gastroenterol.* **26**:393–397.
12. Deltenre, M., and E. de Koster. 2000. How come I've got it? (a review of *Helicobacter pylori* transmission). *Eur. J. Gastroenterol. Hepatol.* **12**:479–482.
13. De Reuse, H., A. Labigne, and D. Mengin-Lecreux. 1997. The *Helicobacter pylori ureC* gene codes for a phosphoglucosamine mutase. *J. Bacteriol.* **179**:3488–3493.
14. Dewhirst, F. E., J. G. Fox, and S. L. W. On. 2000. Recommended minimal standards for describing new species of the genus *Helicobacter*. *Int. J. Syst. Evol. Microbiol.* **50**:2231–2237.
15. Engstrand, L. 2001. *Helicobacter* in water and waterborne routes of transmission. *J. Appl. Microbiol.* **90**:80–84.

16. Feldman, R. A. 2001. Epidemiologic observations and open questions about disease and infection caused by *Helicobacter pylori*, p. 29–51. In M. Achtman and S. Suerbaum (ed.), *Helicobacter pylori: molecular and cellular biology*. Horizon Scientific Press, Wymondham, United Kingdom.
17. Giannino, V., M. Santagati, G. Guardo, C. Cascone, G. Rappazzo, and S. Stefani. 2003. Conservation of the mosaic structure of the four internal transcribed spacers and localization of the *rrn* operons on the *Streptococcus pneumoniae* genome. *FEMS Microbiol. Lett.* **223**:245–252.
18. Groote, D. D., F. Haesebrouck, L. J. V. Doorn, P. Vandamme, and R. Ducatelle. 2001. Evaluation of a group-specific 16S ribosomal DNA-based PCR for detection of *Helicobacter bizzozeronii*, *Helicobacter felis*, and *Helicobacter salomonis* in fresh and paraffin-embedded gastric biopsy specimens. *J. Clin. Microbiol.* **39**:1197–1199.
19. Hassall, E. 2001. Peptic ulcer disease and current approaches to *Helicobacter pylori*. *J. Pediatr. Gastroenterol.* **138**:462–468.
20. Hegarty, J., M. Dowd, and K. H. Baker. 1999. Occurrence of *Helicobacter pylori* in surface water in the United States. *J. Appl. Microbiol.* **87**:697–701.
21. Hobbie, J. E., R. J. Daley, and S. Jasper. 1977. Use of Nucleopore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* **33**:1225–1228.
22. Hopkins, R. J., and J. G. Morris. 1994. *Helicobacter pylori*: the missing link in perspective. *Am. J. Med.* **31**:265–277.
23. Horiuchi, T., T. Ohkusa, M. Watanabe, D. Kobayashi, H. Miwa, and Y. Eishi. 2001. *Helicobacter pylori* DNA in drinking water in Japan. *Microbiol. Immunol.* **45**:515–519.
24. Hulten, K., H. Enorh, T. Nystrom, and L. Engstrand. 1998. Presence of *Helicobacter* species DNA in Swedish water. *J. Appl. Microbiol.* **85**:282–286.
25. Kabir, S. 2001. Detection of *Helicobacter pylori* in faeces by culture, PCR and enzyme immunoassay. *J. Med. Microbiol.* **50**:1021–1029.
26. Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace. 1985. Rapid determination of 16S ribosomal sequences for phylogenetic analysis. *Proc. Natl. Acad. Sci. USA* **82**:6955–6959.
27. Lindkvist, P., F. Enquessellie, D. Asrat, I. Nilsson, L. Muhe, and J. Giesecke. 1999. *Helicobacter pylori* infection in Ethiopian children: a cohort study. *Scand. J. Infect. Dis.* **31**:475–480.
28. Lu, J. J., C. L. Perng, R. Y. Shyu, C. H. Chen, Q. Lou, S. K. F. Chang, and C. H. Lee. 1999. Comparison of five PCR methods for detection of *Helicobacter pylori* DNA in gastric tissues. *J. Clin. Microbiol.* **37**:772–774.
29. Lu, Y., T. E. Redlinger, R. Avitia, A. Galindo, and K. Goodman. 2002. Isolation and genotyping of *Helicobacter pylori* from untreated municipal wastewater. *Appl. Environ. Microbiol.* **68**:1436–1439.
30. Mai, U. E. H., M. Shahamat, and R. R. Colwell. 1990. Survival of *Helicobacter pylori* in the environment, p. 91–96. In H. Menge (ed.), *Proceedings of the Second International Symposium on Helicobacter pylori*. Springer-Verlag, Berlin, Germany.
31. Malaty, H. M., N. D. Logan, D. Y. Graham, and J. E. Ramchatesingh. 2001. *Helicobacter pylori* infection in preschool and school-aged minority children: effect of socioeconomic indicators and breast-feeding practices. *Clin. Infect. Dis.* **32**:1387–1392.
32. Mazari-Hiriart, M., Y. Lopez-Vidal, and Y. Y. Calva. 2001. *Helicobacter pylori* in water systems for human use in Mexico City. *Water Sci. Technol.* **43**:93–98.
33. Mijs, W., K. De Veeese, A. Devos, H. Pottel, A. Valgaeren, C. Evans, J. Norton, D. Parker, L. Rigouts, F. Portaels, U. Reischl, S. Watterson, G. Pfyffer, and R. Rossau. 2002. Evaluation of a commercial line probe assay for identification of *Mycobacterium* species from liquid and solid culture. *Eur. J. Clin. Microbiol. Infect. Dis.* **21**:794–802.
34. Mora, D., G. Ricci, S. Guglielmetti, D. Daffonchio, and M. G. Fortina. 2003. 16S-23S rRNA intergenic spacer region sequence variation in *Streptococcus thermophilus* and related dairy streptococci and development of a multiplex ITS-SSCP analysis for their identification. *Microbiology* **149**:807–813.
35. Mukhopadhyay, B., E. F. Johnson, and R. S. Wolfe. 1999. Reactor-scale cultivation of the hyperthermophilic methanarchaeon *Methanococcus jannaschii* to high cell densities. *Appl. Environ. Microbiol.* **65**:5059–5065.
36. Rahman, M., A. K. Mukhopadhyay, S. Nahar, S. Datta, M. M. Ahmad, S. Sarker, I. M. Masud, L. Engstrand, M. J. Albert, G. B. Nair, and D. E. Berg. 2003. DNA-level characterization of *Helicobacter pylori* strains from patients with overt disease and with benign infections in Bangladesh. *J. Clin. Microbiol.* **41**:2008–2014.
37. Sasaki, K., Y. Tajiri, M. Sata, Y. Fujii, F. Matsubara, M. Zhao, S. Shimizu, A. Toyonaga, and K. Tanikawa. 1999. *Helicobacter pylori* in the natural environment. *Scand. J. Infect. Dis.* **31**:275–279.
38. Shahamat, M., U. Mai, C. Paszko-Kolva, M. Kessel, and R. R. Colwell. 1993. Use of autoradiography to assess viability of *Helicobacter pylori* in water. *Appl. Environ. Microbiol.* **59**:1231–1235.
39. Shahinian, M. L., D. J. Passaro, D. L. Swerdlow, E. D. Mintz, M. Rodriguez, and J. Parsonnel. 2000. *Helicobacter pylori* and epidemic *Vibrio cholerae* O1 infection in Peru. *Lancet* **355**:377–378.
40. Song, Y., C. Liu, D. Molitoris, T. J. Tomzynski, M. McTeague, E. Read, and S. M. Finegold. 2002. Use of 16S-23S rRNA spacer-region (SR)-PCR for identification of intestinal clostridia. *Syst. Appl. Microbiol.* **25**:528–535.
41. Suerbaum, S., and P. Michetti. 2002. *Helicobacter pylori* infection. *N. Engl. J. Med.* **347**:1175–1186.
42. Thomas, J. E., A. Dale, M. Harding, W. A. Coward, T. J. Cole, and J. Weaver. 1999. *Helicobacter pylori* colonization in early life. *Pediatr. Res.* **45**:218–223.
43. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix-choice. *Nucleic Acids Res.* **22**:4673–4680.
44. Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzgerald, N. Lee, M. D. Adams, J. C. Venter, et al. 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**:539–547.
45. Velazquez, M., and J. M. Feirtag. 1999. *Helicobacter pylori*: characteristics, pathogenicity, detection methods and mode of transmission implicating foods and water. *Int. J. Food Microbiol.* **53**:95–104.
46. Westblom, T. U., S. J. Czinn, and C. S. Nedrud (ed.). 1999. Current topics in microbiology and immunology, vol. 241. Gastrointestinal disease and *Helicobacter pylori*: pathophysiology, diagnosis and treatment. Springer-Verlag, Berlin, Germany.